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The primary of this Phase I program was to provide a stable peroral controlled delivery system for a vaccine which was designed to release its contents in the intestinal tract with or without an immunoadjuvant. A single administration of the vaccine must release at a rate sufficient for innoculation of the host immune system for up to six months. To insure sustained release in an environment as dynamic as the intestinal mucosa, adherence to the host tissue for several days is essential. Adhesion of this magnitude will require a delivery system configured with a bioadhesive polymer.

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Ronald R. [illegible] 12/6/95

PI - Signature Date

SYSTEMS

4.0 - TABLE OF CONTENTS

5.0 - INTRODUCTION.

Most current vaccination programs require multiple sub-cutaneous administrations of the vaccine at specific intervals over a period of days or weeks to insure maximum immunization. This vaccination regimen, if to be efficacious, mandates absolute patient compliance, as well as a great deal of time and logistic planning by medical personnel. Additionally, parenteral administration of the vaccine by medical personnel considerably increases the costs associated with the vaccination program.

Subsequent to administration of a vaccine into the bloodstream, only a small portion of the soluble antigen becomes involved in the induction of the immune response; the rest is removed from the body by natural clearance. The process of antigen degradation in the plasma can be differentiated into three phases.

First, during the equilibrium phase, the injection of a soluble antigen is normally followed by a rapid diffusion of the antigen into extravascular spaces and the initial high concentration of the antigen in the plasma drops rapidly.

During second phase, metabolic decay phase, the antigen circulating in the bodily fluids is gradually degraded and its concentration drops slowly.

Third, immune elimination phase begins as soon as antibodies against the antigen begin to be produced, they bind the antigen and the ensuing soluble antigen-antibody complexes are taken up and digested by macrophages. Rapid decline in the concentration of free antigen follows. If antibodies are already present in the plasma at the time of administration, the concentration of the antigen drops rapidly without a noticeable metabolic decay phase.

With subsequent booster doses of the vaccine, the length of the metabolic decay phase is increased. This increase provides for a greater antigen retention time in the body and results in greater antibody production.

Most current vaccine regimens are administered parenterally. However, parenteral administration of the vaccine by medical personnel considerably increases the costs associated with the vaccination program. This vaccination regimen, if to be efficacious, mandates absolute patient compliance, as well as a great deal of time and logistic planning by medical personnel. Therefore, vaccination programs which could be administered efficaciously by a single dose in an oral formulation are desirable.

Conventional fast release oral preparations deliver drug contents all at once for adsorption into the body. This can lead to high peak drug levels. If the drug is excreted at a rapid rate the levels decline below therapeutic level within a few hours, thereby necessitating frequent dosing.

In the design of an oral sustained release drug delivery system, there are a number of in-vitro and in-vivo aspects involved during the development phase. The physicochemical properties such as, solubility, pKa, stability, type of delivery system and selection of appropriate excipients, processability, mechanism of release and evaluation of drug release rate are critical design considerations.

The primary objective of the Phase I program was to provide a stable peroral controlled release delivery system for a vaccine which was designed to release its contents in the intestinal tract with or without an immunoadjuvant. Ultimately, a single administration of the vaccine must release at a rate sufficient for inoculation of the host immune system and for a duration of at least six months. To insure sustained release in an environment as dynamic as the intestinal mucosa, adherence to the host tissue for several days is essential. Adhesion of this magnitude requires a delivery system configured with a bioadhesive polymer that integrates the surface of the microcapsules and the mucosa. SBIR Phase I Program efforts focused on the development of the most feasible method(s) for encapsulating inactivated Staphylococcus enterotoxin B (SEB), as a model vaccine, in a stable matrix which allows for sustained release in the gut.

More specifically, the goal of this research effort was to provide the user community with a vaccination delivery system that provides maximum immunization from a single oral dose and reduces the time and logistical demands of the trained medical personnel.

SYSTEMS

6.0 - PHASE I CONTRACT No. DAMD17-95-C-5066.

6.1 Phase I Objective.

The primary objective of the Phase I program was to provide a stable peroral controlled release delivery system for a vaccine which was designed to release its contents in the intestinal tract with or without an immunoadjuvant. Ultimately, a single administration of the vaccine should release at a rate sufficient for inoculation of the host immune system and for a duration of at least six months. To insure sustained release in an environment as dynamic as the intestinal mucosa, adherence to the host tissue for several days is essential. Adhesion of this magnitude required a delivery system configured with a bioadhesive polymer that integrates the surface of the microcapsules and the mucosa. SBIR Phase I Program efforts focused on the development of the most feasible method(s) for encapsulating inactivated Staphylococcus enterotoxin B (SEB), as a model vaccine, in a stable matrix which allows for sustained release in the gut.

During the Phase I research effort, Chemical Delivery Systems, Inc. (CDS) constructed a delivery system which was engineered to be administered orally to provide the user with the sustained release of a vaccine for the period of time necessary for maximum immunization from a single administration. However, it was concluded that vaccine bioavailability and efficacy of the delivery system needed to be demonstrated before an oral preparation was prepared. Therefore, because it is extremely difficult to dose an animal, such as mice, orally it was determined by CDS, Inc.'s consultants that delivery system efficacy could be demonstrated by an intraperitoneal injection. More specifically, the goal of this program was to provide the user community with a single dose vaccination delivery system that reduces the time and logistical demands of the trained medical personnel, without sacrificing maximum immunization.

6.2 Phase I Approach

The delivery system developed by CDS, Inc., during the Phase I effort, consisted of microspheres composed of a biocompatible polymer matrix which was designed to degrade at a consistent rate; releasing the imbedded SEB toxoid.

Due to the large molecular size (27,000 DMW) of biologically active agents, such as Staphylococcus B enterotoxins (SEB) vaccines, diffusion through polymer walls could not be relied upon as an effective delivery mechanism. Instead, the delivery mechanisms which entrap the SEB toxoid, consists of biodegrading polymer matrices and would provide a means for sustained, predictable dosages over an extended period of time. The polymers which were considered for the matrix of the microspheres must degrade at a consistent and predictable rate and be biocompatible. The polymers utilized in this controlled release system must also have rates of degradation that can be modified to sustain the release rates for the entire immunization period of various vaccinations. In particular, the Phase I research effort tested polymers that were designed to degrade at a rate that sustains the release of the enterotoxin over several days so as to insure immunization of the host to the SEB.

Several suitable copolymers were considered for incorporation into a controlled release delivery system: Poly(Lactide-co-Glycolide)(PLG), Poly(3-hydroxybutyrate)(PHB), Poly(3-hydroxy-valeric acid)(PHV) and a PHB/PHV copolymer. PLG copolymers have been used in the sustained release of peptide hormones and many other therapeutic applications. When exposed to water, aqueous acidic or basic solutions, PLG hydrolyses to form glycolic acid and lactic acid monomers. The rate of this biodegradation is dependent on the copolymer ratio of lactide/glycolide, as well as other properties. The lactide/glycolide polymers are commercially available in several grades, as is the PHB and PHV.

By reabsorbing completely in vivo,PLG leaves no residual polymer matrices. PHB is an aliphatic polyester that is synthesized by bacteria (*Alcaligenes eutrophus*) and is completely biodegradable. Through biodegradation, PHB can be converted to hydrolyzable monomers that can be reduced to carbon dioxide, water and energy by bacteria. The rate of degradation is dependent on the degree of exposure to microbes and the availability of environmental factors, such as oxygen, that encourage their colonization on the polymer. PHV is an aliphatic polyester that is synthesized along with PHB and has similar physical properties including comparable degradation rates. A broad range of PHB/PHV copolymers are available and can be manufactured from the these compounds with varying levels of crystallinity and melting points.

To prohibit degradation of the microspheres during the prolonged exposure to acidic gastric juices prior to the intestinal tract, an enteric coating was applied to the microspheres. These polymer coatings rapidly dissolve and release the microspheres in the more neutral solutions of the small intestines (pH 5.5-6.5). Various enteric polymers were tested; these were [cellulose acetate phthalate (CAP), cellulose acetate trimellitate (CAT), hydroxypropyl methylcellulose pthalate (HPMCP), cellulose acetate succinate (CAS) and polyvinyl acetate (PVA)] to insure maximum protection from acid gastric juices, optimum release in the small intestines and compatibility with bioadhesive polymers.

To insure that the host immune system received prolonged vaccine exposure necessary for inoculation, a bioadhesive material was integrated into the microspheres. Upon contact with the intestinal lining, a bioadhesive polymer should generate sufficient interfacial bonds between the mucosa and the microspheres to enable adhesion to the lining of the small intestine for several days. Several polycarboxilic acids (i.e. Carbomer(Carbopol 934), Polycarbophil(Carbopol EX-55, Noveon AA-1), Carboxymethylcellulose, Polycarbopol, Polyacrylic acid, Sodium Alginate and Hydroxypropylcellulose) were evaluated and considered as candidate bioadhesives due to their polyanionic characteristics. High molecular weight polycationic compounds such as Chitosan, Polybrene and cationic gelatin have also demonstrated adhesive interaction with mucin. These polymers swell in water allowing their polymer chains to entangle with mucin on the surface of the tissue and form hydrogen bonds between the unionized carboxylic acid groups and the mucin.

6.3 - SUMMARY OF PHASE I STUDIES.

6.3.1 - Literature Review

During the first month of the subject contract research effort, CDS, Inc. performed an extensive database search in order to locate suitable bioadhesives for this application. A list of bioadhesive literature references is located in Section 8.0.1 of this report.

In order to insure that the host immune system receives prolonged vaccine exposure necessary for effective inoculation, a bioadhesive material was designed to be integrated into the candidate microspheres. Upon contact with the intestinal mucosa, the bioadhesive polymer could generate sufficient interfacial bonds between the mucosa and the SEB-toxoid microspheres to enable adhesion to the lining of the small intestine for several days.

Additionally, the candidate bioadhesive polymers swell in water allowing their polymer chains to entangle with mucin on the surface of the tissue and form hydrogen bonds between the unionized carboxylic acid groups and the mucin. This chemical bonding allows the SEB-toxoid microspheres to remain within the small intestine and biodegrade over an extended period of time.

CDS, Inc. also performed a database search for the identification of polymers suitable for this application. Historically, controlled delivery of pharmaceuticals has been achieved through the utilization of polylactide and glycolide (PLG) polymers. The two most common systems based on biodegradable polymers are microspheres and implants. The principal mode of degradation for PLG polymers and copolymers is hydrolysis. Degradation proceeds first by diffusion of water into the material followed by random hydrolysis, fragmentation of the material and finally a more extensive hydrolysis accompanied by phagocytosis, diffusion and metabolism. Hydrolysis is affected by the size and hydrophilicity of the particular polymer, the crystallinity of the polymer and the pH and temperature of the environment. Much work has centered on the preparation of injectable or implantable drug delivery devices from polyesters synthesized from lactide and glycolide monomers. A major disadvantage of synthetic polymers is that the residues of the polymerization catalyst must be reckoned with.

Alternative biodegradable materials, such as polyhydroxybutyrate (PHB), are available, but have received relatively little attention. PHB s a linear polyester produced from D(-) β-hydroxybutyric acid by various bacteria. A list of references is listed in Section 8.0.2 of this report.

6.3.2. Vaccine Inactivation

CDS, Inc.'s program consultants, Dr. Nancy Bigley, Wright State University, and Dr. Raul Riser, Toxin Technologies, determined that the originally proposed formalin SEB inactivation procedure would pose considerable delays in achieving the Phase I research goals.

As originally proposed by CDS, Inc., the SEB was to be inactivated prior to encapsulation by utilizing a 0.1 - 0.2% formaldehyde solution, adjusted to pH 6-9, and maintained at 37°C for 1-3 weeks. However, it was determined, by CDS' consultants, that SEB inactivated utilizing formaldehyde inactivation procedures may automatically become reactive after relatively short periods of storage (less than one week).

Secondly, the proposed formaldehyde toxoiding procedure requires a considerable incubation period in order to complete the inactivation. Thus, subsequent mitogenicity testing may indicate that the SEB has not been completely inactivated. Therefore, the toxoid would need to be incubated again to insure complete inactivation. The possibility of repeated, lengthy incubation periods associated with formalin toxiding of the SEB preclude its use during the Phase I research effort.

Subsequently, researchers at CDS, Inc. and Wright State University, as well as the commercial supplier of the SEB, determined that it would be significantly more technically feasible to toxoid the SEB utilizing a well-known glutaraldehyde inactivation method. The purified SEB toxin (55 μg/ml) was incubated at 37°C in 0.11% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 8) for 30 minutes. Lysine was then added to a two-fold molar excess over glutaraldehyde. The resulting toxoid was then dialyzed overnight in a phosphate buffered saline solution. Verification of inactivation was accomplished through the utilization of a commercially available, non-radioactive mitogenicity assay using mouse lymphocytes.

Researchers at CDS and Wright State University believe that the glutaraldehyde toxoiding procedure was more effective at preventing re-activation of the toxoid, as well as accommodating the relatively short time periods associated with this Phase I research effort.

In order to evaluate any differences in inactivation procedure results, researchers at CDS, Inc. toxoided the SEB utilizing formalin inactivation. The SEB was inactivated prior to encapsulation by utilizing a 0.1 - 0.2% formaldehyde solution, adjusted to pH 6-9, and maintained at 37°C for 1-3 weeks. CDS, Inc. filed for, and was denied, a one month extension to the Phase I contract period, in order to complete further testing and evaluation of the formalin inactivated SEB. The SEB toxoid was to be tested for inactivity, through the use of a mitogenicity assay, by Dr. Nancy Bigley, Wright State University. Cellular proliferation was to be measured using a Cell Proliferation ELISA, BrdU (colormetric) kit (Boehringer Mannheim), a non-radioactive alternative to the [^{3}H]-thymidine incorporation assay. 5'bromo-2'-deoxy-uridine, a pyrimidine analogue, was to be incorporated into the DNA of proliferating cells and detected by an immunoassay. The reaction product quantified by measuring the absorbance using a scanned multiwell spectrophotometer.

The results of the glutaraldehyde inactivated sample series is shown graphically in Figure 3 at the end of this report.

6.3.3 Polymer Selection

The polymers being considered for the matrix of the microspheres must degrade at a consistent and predictable rate as well as exhibit biocompatibility. The polymers utilized in this program's controlled release system exhibited rates of degradation that can be modified to sustain the release rates for the entire immunization period of the vaccination. In particular, this study tested polymers that degrade at a rate that sustains the release of the enterotoxin over several days to insure immunization of the host to the SEB.

Several suitable copolymers that were considered for incorporation into a controlled release delivery system are: Poly(Lactide-co-Glycolide)(PLG), Poly(3-hydroxybutyrate)(PHB), Poly(3-hydroxy-valeric acid)(PHV) and a PHB/PHV copolymer. PLG copolymers have been used in the sustained release of peptide hormones and many other therapeutic applications. When exposed to water, aqueous acidic or basic solutions, PLG hydrolyses to form glycolic acid and lactic acid monomers. The rate of this biodegradation is dependent on the copolymer ratio of lactide/glycolide. The lactide/glycolide polymer is commercially available in several grades, as is the PHB and PHV. By reabsorbing completely in vivo,PLG leaves no residual polymer matrices. PHB is an aliphatic polyester that is synthesized by bacteria (*Alcaligenes eutrophus*) and is completely biodegradable. The rate of degradation is influenced by a range of environmental and material parameters and is particularly dependent on the microbial activity of the environment and the surface area of the microcapsule. Other factors influencing the rate of biodegradation are temperature, molecular weight, crystallinity and pH.

6.4 Bioadhesion

It has been extensively documented and studied that certain polymers exhibit bioadhesive properties. Generally, bioadhesive polymers swell in water; thereby allowing their polymer chains to entangle with mucin on the surface of the tissue and form hydrogen bonds between the unionized carboxylic acid groups and the mucin. Bioactive materials designed to be released in the gastrointestinal tract must not: (1) be entrained beyond the desired site of action, (2) be eliminated before they have had a chance elicit an immune response, (3) be eliminated before they have been absorbed into the bloodstream. Polymers designed to produce strong adhesive interactions with biological tissues could be utilized to keep the microspheres in contact with the intestinal epithelium for extended periods of time. However, it is important to realize that the targeted tissue is coated with a continuous layer of mucus. The delivery system was designed to penetrate this protective layer and bond with the underlying epithelium, or adhere to the mucus itself. Since the mucus and the epithelial cells at the surface of the villi are continuously replaced, it would be impossible for the microsphere to adhere permanently to the lumen of the small intestine. Subsequently, the program goal was to ultimately delay movement through the gastrointestinal tract, during which time adhesion could be achieved between the bioadhesive and the target tissue.

6.5 Encapsulation/Studies Procedures

Microencapsulation can be considered to be a specialized form of packaging, in that particulate matter can be individually coated for protection against environmental influences. For the encapsulation system to be effective, the capsules containing the biologically active material(s) must be specifically tailored to the end use application(s). There are presently many distinctly different techniques and processes which are currently available to CDS in order to encapsulate a myriad of chemical and biological compounds. To ascertain concept feasibility, CDS utilized the expansive microencapsulation technology and experience it possesses to select from numerous distinct microencapsulation processes. Most of these processes are categorized under the following classifications:

TABLE 1
Microencapsulation Processes

Simple coacervation	Polymer-polymer incompatibility
Complex coacervation	Phase separation
Meltable dispersion	Solvent exchange
Fluidized coating	Pan coating
Stationary extrusion	Centrifugal extrusion
Spray coating	Liposome and surfactant vesicles
Matrix polymerization	Interfacial polymerization
In-situ polymerization	Solvent evaporation

When considering these polymer-dependent processes (both physical and/or chemical), each have their own respective advantages and disadvantages. Each encapsulation process has the potential to impart unique physical (including the ability to utilize systems yielding a wide range of particle sizes) and chemical characteristics tailored to a specific application. The resultant encapsulation process selection is highly dependent upon the material to be encapsulated, its chemical/physical properties, the sensitivity of its structures to various solvent classes, candidate polymers to be used as the microcapsule (or microsphere) wall material, and the desired performance specifications of the final formulation.

In this program, initial efforts focused on microsphere systems that embed the Staphylococcus B enterotoxin in a polymer matrix. These included solvent evaporation and matrix polymerization with copolymers of Poly(lactide/glycolide) and PHB as the encapsulating polymer since this process has already shown to be successful in the encapsulation of biologically active compounds (i.e. Luteinizing hormone) and limited success with respect to the encapsulation of vaccines. However, due to the limited success of these prior research efforts, CDS will investigated other suitable encapsulation processes, when sufficient physical and chemical property data of the specific enterotoxin could be researched and defined (e.g. solvent/process compatibility). Ultimately, CDS, Inc. investigated the compatibility of homopolymers and copolymers of PHB/PHV as candidate matrix polymers for this system. Additionally, CDS, Inc. proceeded with encapsulation studies utilizing those processes which were determined to be most amenable to the proposed research program. The toxoided SEB was added to a solution of PHB in a volatile organic solvent. The mixture was then emulsified in water and agitated at a constant rate until the solvent was no longer present. The resulting microspheres were washed, isolated by filtration and dried.

The initial encapsulation feasibility studies included the analysis and decision-making process of pertinent microsphere parameters which directly affected the end use performance. These included the encapsulating polymer, its molecular weight, degree of crystallinity, the specific payload formulation, the desired particle size, payload : polymer ratio (determines wall thickness), solvent selection, solvent compatibility with the Staphylococcus B Enterotoxin, and finally, processing conditions suitable for microencapsulation.

The ability to tailor the microspheres for a specific, unique application required the manipulation of many independent variables. Some principle properties are listed below:

TABLE 2

Microcapsule Characteristics

Microcapsule matrix material
Microcapsule particle size
Percentage of active ingredients
Microsphere degradation rate
Integrity of the enteric coating
Vaccine concentration in titer
Adherence of bioadhesive polymers

The properties of these independently controlling variables are described as follows:

Microsphere matrix material:

Candidate microsphere matrix or wall material is referred to as that material capable of suspending active materials (liquid, solid or semisolid) while providing the previously established desired properties. Phase I research included work targeted toward selecting additional polymers, such as PHB/PHV with properties similar to those of Poly(lactide-glycolide) copolymers which were demonstrated to be biocompatible and biodegradable with the gastrointestinal tract and which may also be suitable for the microencapsulation of other potential vaccines.

Both natural and synthetic polymers are commonly utilized as wall materials. Furthermore, polymer selection is only somewhat dependent upon the physical characteristics of the candidate material to be microencapsulated. The candidate biological material can exhibit either a liquid or solid form or can be incorporated as an admix with a suitable carrier vehicle.

Consequently, the variety of wall materials and microencapsulation processes available to the investigator gave considerable latitude with respect to the types of material that can be successfully microencapsulated.

Microsphere particle size:

Microspheres can be constructed in sizes ranging from one micrometer in diameter up to 1000 micrometers. Macrospheres can also be constructed ranging in sizes spanning several centimeters. The sphere size and mean size distribution can be reasonably controlled and are highly reproducible in most encapsulation systems.

Percentage of active ingredients:

A typical percent loading achieved utilizing various microencapsulation processes ranges from approximately 1 to 50%. These values correspond to percentage of the matrix occupied by the active component. As the percentage of matrix material decreases the degree of protection that the matrix polymer can provide for the active material decreases and the incidence of active material imbedded in the sphere surface increases. The resultant phase ratio, in addition to the selected wall material, will dictate the mechanism of release.

Physical form of microsphere:

The microencapsulated biological compounds can be isolated as dry, free-flowing powders, or they can exist in the form of slurry. Additionally, microspheres can be constructed to bond together as a self-sustaining mass which can subsequently be shaped into tablets, capsules or cakes. The physical form of the microspheres was engineered to yield optimum effectiveness for the presently proposed system.

Integrity of the enteric coating:

During the proposed Phase I research program, there existed a critical need for the utilization of enteric polymers which exhibit either minimal or no permeability to gastric acidic solutions through the initial portion of the gastrointestinal tract. These enteric coatings must degrade once the microspheres have reached the more neutral solutions (pH 5.5-6.0) of the small intestines to expose the bioadhesive polymers for adhesion to the intestinal wall.

Microsphere degradation rate:

The inherent rate of degradation of the microsphere matrix material is one of the most important physical characteristics associated with many microencapsulation applications. The utilization of specific polymers for the matrix material which exhibit the desired permeability characteristics will facilitate the development of the proposed delivery system.

Concentration of vaccine titer:

The effective dosage of SEB for practical inoculation by this single administration delivery system has as yet to be determined. Calibration of vaccine titer and dosage of microspheres for effective inoculation would be determined in a Phase II project.

Adherence of the bioadhesive:

To insure inoculation from a single administration of the SEB vaccine, the voyage of the microspheres through the gastrointestinal tract must be delayed in the small intestine to release the vaccine over several days as the microsphere degrades. By integrating a bioadhesive polymer into the polymer matrix, the microsphere will be tethered in the small intestine to provide this necessary delay. Determining the period of necessary adhesion will be effected by the rate of degradation and the inoculation period that is necessary for the SEB vaccine. The bioadhesive polymer must provide biocompatability and biodegradation rates similar to the other components in the encapsulation system. In addition, the bioadhesive polymer must be compatible with the encapsulating matrix polymer and the solvent(s) utilized in the encapsulation process.

Microencapsulation of the SEB toxoid under the Phase I research effort was conducted at CDS' facility in a Baker SterilGard biological hood. The biological hood is equipped with a vacuum hood and a spill resevior basin. Halon fire extinguishers are located through out the laboratories. Even though the Staphylococcus enterotoxin toxoid is non pathogenic, all CDS personnel involved in the Phase I effort are required to wear full length laboratory coats, surgical gloves ,filter masks and protective eyewear.

6.6 Animal Evaluations

The efficacy of CDS' controlled release vaccine delivery system was tested by administering the Staphylococcus B enterotoxin vaccine to young adult BALB/C mice (8 to 9 weeks old). The mice received approximately 0.25ml of microencapsulated preparations of vaccine via hypodermic syringe fitted with a twenty-three gauge needle.

The mice were maintained and tested in the animal care facilities of Wright State University in Fairborn, Ohio. Wright State University is fully-accredited by the American Association of Laboratory Animal Care (AAALAC). The Phase I program protocol and procedures were reviewed and approved by the Wright State University Laboratory Animal Care and Use Committee (LACUC). Fecal samples and sera (collected via bleeding halothane-anaesthetized mice from the preorbital plexus) were collected from the mice 7 and 14 days after the injection of the toxoided SEB-containing microcapsules to measure for antibody response from the mice.

7.0 - PHASE I CONCLUSIONS

During the Phase I research period, CDS, Inc. transmitted several samples of microencapsulated SEB toxoid to Wright State University for testing and evaluation. Stimulation of mitogenicity, as compared with unencapsulated control samples, and animal studies in order to determine frequencies of T-cells expressing cytokines which favor antibody production were studied during the research effort. In addition to stimulating specific reactive T-cells, SEB is known to induce production of IFN-γ. Concentrations of this cytokine were measured using an IFN-γ specific Enzyme Linked Immunosorbent Assay (ELISA). Capture and detection antibodies specific for IFN-γ were used to develop this assay. Culture supernatants were assayed for production of IFN-γ and concentrations quantified by comparing to a standard curve generated with recombinant murine IFN-γ.

The encapsulated inactivated SEB samples were prepared using the polymers listed in Table 3 below. The toxoided SEB was added to a solution of the candidate polymer in a volatile organic solvent. The mixture was then emulsified in water and agitated at a constant rate until the solvent was no longer present. The resulting microspheres were washed, isolated by filtration and dried.

Candidate Encapsulation Polymers
Table 3

Poly Lactide-Glycolide Polymers
Polyhyroxybutarate (PHB) Polymer
Polyhydroxybutrate(PHB)/polyhydroxyvalerate(PHV) Polymer

Poly Lactide-Glycolide (PLG) copolymers have been historically used in the sustained release of peptide hormones and many other therapeutic applications. When exposed to water, aqueous acidic or basic solutions, PLG hydrolyses to form glycolic acid and lactic acid monomers. The rate of this biodegradation is dependent on the copolymer ratio of lactide/glycolide. The lactide/glycolide polymer is commercially available in several grades, as is the PHB and PHV. The primary disadvantage of synthetic polymers is that residues of polymerization catalysts must be reckoned with. By reabsorbing completely in vivo,PLG leaves no residual polymer matrices. PHB is an aliphatic polyester that is synthesized by bacteria and is completely biodegradable. Through biodegradation, PHB can be converted to hydrolyzable monomers that can be reduced to carbon dioxide and energy by bacteria. The rate of degradation is dependent on the degree of exposure to microbes and the availability of environmental factors, such as oxygen, that encourage their colonization on the polymer. PHV is an aliphatic polyester that is synthesized along with PHB and has similar physical properties including comparable degradation rates. A broad range of PHB/PHV copolymers are available and can be manufactured from these compounds with varying levels of crystallinity and melting points.

After encapsulating the toxoided SEB, the capsules were suspended in a bioadhesive polymer solution. Noveon AA-1, polycarbophil was utilized at a concentration of 0.2% in order to achieve bioadhesion to the intestinal mucosa. Polycarbophils are polymers of acrylic acid crosslinked with polyalkenyl ethers or divinyl glycol.

To insure that the host immune system received prolonged vaccine exposure necessary for inoculation, a bioadhesive material was integrated into the microspheres. Upon contact with the intestinal lining, a bioadhesive polymer should generate sufficient interfacial bonds between the mucosa and the microspheres to enable adhesion to the lining of the small intestine for several days. Several polycarboxilic acids (i.e. Carbomer(Carbopol 934), Polycarbophil(Carbopol EX-55, Noveon AA-1), Carboxymethylcellulose, Polycarbopol, Polyacrylic acid, Sodium Alginate and Hydroxypropylcellulose) were evaluated and considered as candidate bioadhesives due to their polyanionic characteristics. High molecular weight polycationic compounds such as Chitosan, Polybrene and cationic gelatin have also demonstrated adhesive interaction with mucin. These polymers swell in water allowing their polymer chains to entangle with mucin on the surface of the tissue and form hydrogen bonds between the unionized carboxylic acid groups and the mucin.

The efficacy of CDS' controlled release vaccine delivery system was tested by administering the inactivated Staphylococcus B enterotoxin vaccine to young adult BALB/C mice (8 to 9 weeks old). The mice received approximately 0.25ml of microencapsulated preparations of vaccine via hypodermic syringe fitted with a twenty-three gauge needle.

During the SBIR Phase I research program, researchers at CDS, Inc. concluded that the glutaraldehyde-inactivated SEB (GI-SEB) did not stimulate mitogenicity in spleen cells from BALB/c mice as measured in a cell proliferation assay. The active SEB did stimulate mitogenicity in spleen cells from BALB/c mice. Figure 1, Appendix, details the absorption patterns of 24, 48 and 72 hour cultured murine splenocytes exposed to various concentrations of SEB. All cultures show an increase in cellular proliferation over a 72 hour period. Figure 2, Appendix, shows absorbance values of 24, 48 and 72 hour cultured murine splenocytes exposed to various concentrations of glutaraldehyde-inactivated SEB. The toxoided SEB did not stimulate cellular proliferation over a 72 hour period. In contrast to the effect of SEB, GI-SEB also did not stimulate spleen cells from this mouse strain to produce interferon-γ (IFN-γ) at 24 and 48 hours post exposure. Table 4 of the Appendix shows the IFN-γ concentrations of murine spleen cell culture supernatants exposed to various concentrations of SEB. Table 5 of the Appendix shows the IFN-γ concentrations of murine spleen cell culture supernatants exposed to various concentrations of GI-SEB. No IFN-γ was detected in any culture supernatants, even those exposed to 10μg/ml concentrations of the toxoided SEB. It was anticipated that BALB/c mice immunized with GI-SEB would exhibit higher antibody titers to SEB by ELISA than did mice immunized with SEB at 2 and 3 weeks following immunization. Subsequent to IP injection and ELISA, the encapsulated inactivated SEB demonstrated anti-body titers to SEB at much lower levels than was expected. The anti-body titers were greater than control levels but lower than active SEB anti-body titer levels. Upon review of the immunization of the BALB/c mice, it has been concluded that much of the sample remained aggregated in the tip of the syringe. Subsequently, the mice may have received a much lower dose of the SEB vaccine than was originally determined. Additionally, one would expect lower antibody titers to SEB from the microencapsulated inactivated SEB sample due to the fact that the toxin was available only as the microcapsules were degrading.

The following procedure describes the animal studies for antibody production, which was used for the glutaraldehyde inactivated SEB and may be used for the formaldehyde toxoided SEB.

Ten week old female BALB/c mice were split into four groups consisting of 5 mice per group. The 4 groups consisted of the following: PBS control, SEB-active, SEB Toxoid, and encapsulated SEB toxoid. All preparations were at a concentration of 50μg/.2ml and .2ml/mouse was the injected dose for each preparation. 19 days post-injection, mice were anesthetized and bleed via a terminal heart stick procedure. A 23 gauge needle and syringe were used to collect blood. The pooled blood was collected and placed in microtainer brand serum separator tubes. The tubes were centrifuged for 2 minutes at 7000 g's. Serum was collected and stored at -70°C.

Serum samples were assayed for antibody production by ELISA. Purified SEB was diluted in $NaHCO_3$ to a final concentration of 2μg/ml. 50μg was added to each well of a 96 well ELISA plate and incubated at 4°C overnight. The plate was washed and blocked with PBS/10%CS for 2 hours at room temperature. Again the plate was washed and 50μl samples and controls were added to the appropriate wells and incubated at 4°C overnight. Following wash, biotin goat anti-mouse IgG (H+L) antibody was diluted 1:50,000 and added at 100μl/well for 1 hour at room temperature. The plate was washed to remove primary antibody and incubated with a 1:300 concentration of avidin-peroxidase for 30 minutes at room temperature. Finally, ABTS substrate solution was added to the washed plate and incubated for 20 minutes at room temperature. The optical densities of each well were assessed at 450nm using a Vmax ELISA reader. Control well were incubated with rabbit anti-toxin and detected with biotin goat anti-rabbit IgG (H+L) antibody.

The results of the glutaraldehyde inactivated sample series is shown graphically in Figure 3, Appendix, at the end of this report.

Additionally, in order to evaluate any differences in inactivation procedure results, researchers at CDS, Inc. are toxoiding the SEB utilizing formalin inactivation. The SEB was inactivated prior to encapsulation by utilizing a 0.1 - 0.2% formaldehyde solution, adjusted to pH 6-9, and maintained at 37°C for 1-3 weeks. CDS, Inc. filed for, but unfortunately was denied, a one month extension to the Phase I contract period, in order to complete further testing and evaluation of the formalin inactivated SEB.

Upon recommendation from Program Management, CDS, Inc. suspended SEM evaluation of the glutaraldehyde inactivated SEB samples until testing and evaluation of the formalin inactivated samples could be completed. Unfortunately, CDS, Inc. was denied a contract extension which would have provided additional time for this research effort.

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Figure 1 Cellular Proliferation Induced by Staphylococcal Enterotoxin B (SEB)

9.0 - Appendix

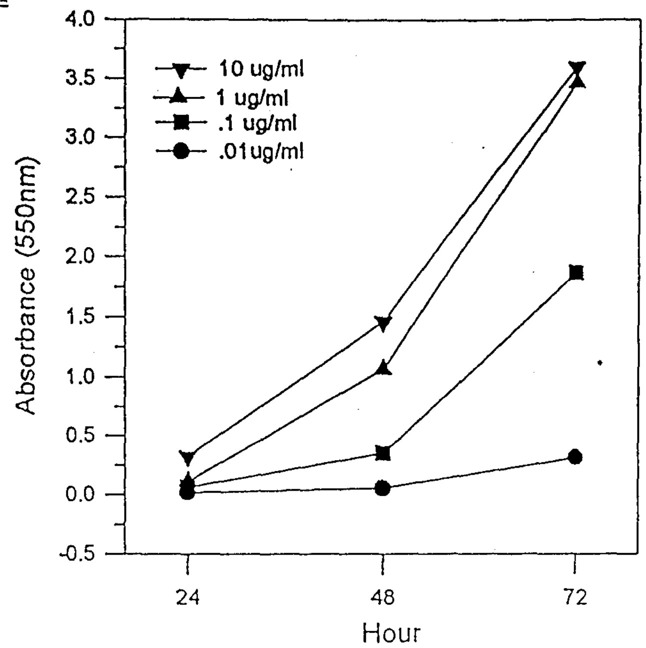
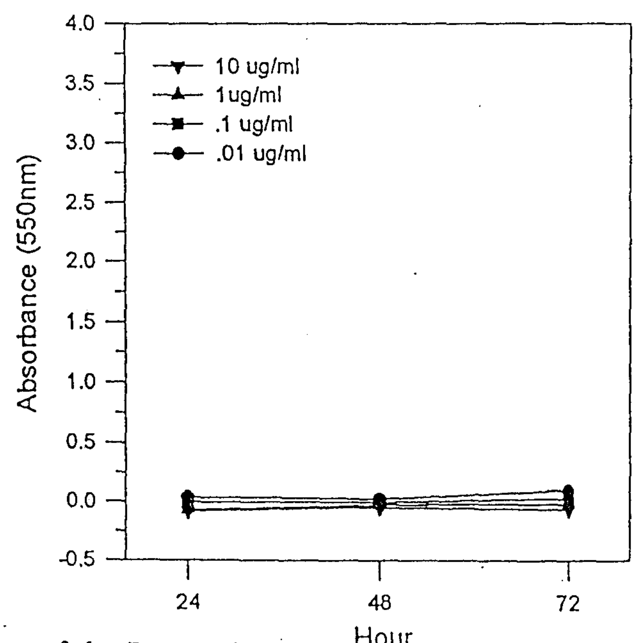


Figure 2 Cellular Proliferation Not Induced by Glutaraldehyde-Inactivated Staphylococcal Enterotoxin B (GI-SEB)



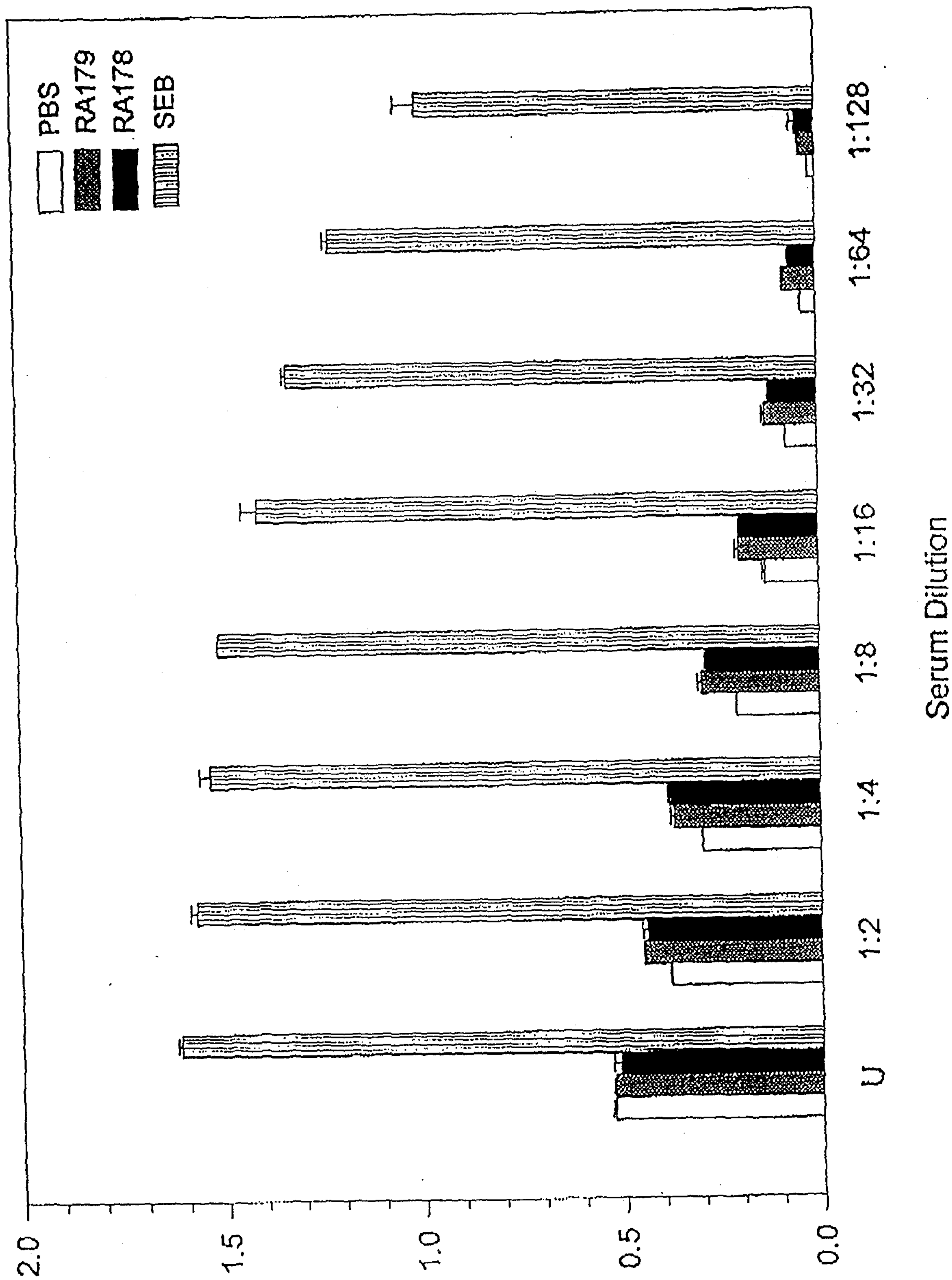


Figure 3 Anti-staphylococcal enterotoxin B (SEB) antibody production in PBS, RA179, RA178, or SEB pretreated mice

Table 4 Enhanced IFN-γ Production in Murine Spleen Cell Cultures Upon Stimulation with Staphylococcal Enterotoxin B (SEB)

IFN-γ (ng/ml)			
SEB	Hour		
	24	48	72
10 μg/ml	6.42 ± 0.49	101.77 ± 2.90	85.19 ± 2.20
1 μg/ml	1.30 ± 0.46	51.32 ± 1.92	46.51 ± 0
.1 μg/ml	0	8.78 ± 1.30	26.74 ± 2.19
.01 μg/ml	0	0.17 ± 0.04	1.67 ± 0.01
C*	0	0	0

* Control (C) cultures not cultured with SEB

Table 5 No Production of IFN-γ in Murine Spleen Cell Cultures Upon Stimulation with Glutaraldehyde-Inactivated Staphylococcal Enterotoxin B (GI-SEB)

IFN-γ (ng/ml)			
GI-SEB	Hour		
	24	48	72
10 μg/ml	0	0	0
1 μg/ml	0	0	0
.1 μg/ml	0	0	0
.01 μg/ml	0	0	0
C*	0	0	0

* Control (C) cultures not cultured with GI-SEB

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
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as

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